siRNA as an alternative therapy against viral infections

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Abstract

siRNA (small interfering ribonucleic acid) is a method that can be used to deal with viral infections. The principle of siRNA is based on the complementarity of synthetic dsRNA to an RNA virus, which in consequence, will be silenced. Many studies are currently examining the effects of siRNA on replication of diverse virus types like Hepatitis C, polio and HIV. The choice of the siRNA target sequence is crucial. It has to be very homologous to the target RNA, but it cannot target RNA of the host cell. To reduce the possibility for the virus to escape from the siRNA therapy by mutating, multiple siRNAs have to be used that target different sequences of the viral RNA. siRNA is a promising method to deal with viral infections. The principle of siRNA is based on the complementarity of synthetic dsRNA to an RNA virus which, in consequence, will be silenced. Many studies are currently examining the effects of siRNA on replication of diverse virus types like Hepatitis C, polio and HIV. The choice of the siRNA target sequence is crucial. It has to be very homologous to the target RNA, but it cannot target RNA of the host cell. To reduce the possibility for the virus to escape from the siRNA therapy by mutating, multiple siRNAs have to be used that target different sequences of the viral RNA. Still, siRNA therapy is facing some difficulties such as the specialized delivery to the infected tissue and the siRNA protection from the digestion by nucleases. These problems will have to be solved before siRNA therapy could be used in clinical trials. Based on several researches, siRNA could be used as an alternative therapy against life threatening viruses. This therapy is recommended to be tested further in clinical trials with respect to several aspects, such as design of siRNA and transfer mechanism. (Health Science Indones 2010; 1: 58 - 65)

Key words: siRNA, viral infections, viral target, alternative therapy

The discovery of the RNA interference (RNAi) mechanism took place in 1998 for which a Nobel Prize was awarded. RNAi is a specific gene silencing process based on RNA homology. The homologous RNA sequences form double-stranded RNA (dsRNA). Subsequently, dsRNA triggers destruction and thereby gene activity is suppressed. This process was initially observed in plants as a conserved defense mechanism against viral infections and foreign nucleic acids. After the discovery of the RNAi mechanism, it was used in functional genomics studies of model organisms, Drosophila melanogaster and Caenorhabditis elegans to silence the activity of the gene of interest.

Currently, RNAi has become a powerful tool in medicine as a method to silence genes responsible for cancer and neurodegenerative diseases. Now, a new siRNA application has
arise to become a cure or vaccination against life threatening viruses like HIV or Hepatitis C. In current studies aiming to inhibit the viral replication, small interfering RNA (siRNA) is used in order to guide the RNAi mechanism. The siRNA therapy against viral infections aims to inhibit the viral replication, thereby curing infected tissue, restoring cell functions and limiting the symptoms caused by virus infection. SiRNA can also be used as vaccination, by preventing the entrance of the viral particle into the cell. However, to outline a good siRNA antiviral therapy, the knowledge of the virus life cycle has to be gained.

This article reviews the important aspects of which siRNA therapy has to fulfill to effectively inhibit viral replication. We also review the available research reports including the advantages and disadvantages of the method in targeting the viral infections.

The pathway of siRNA

siRNA duplexes consist of approximately 21 nucleotides and can induce gene silencing through sequence specific cleavage of perfectly complementary messenger RNA (mRNA). In the natural RNAi mechanism, dsRNA is recognized and cleaved by a Dicer enzyme complex into siRNA. Next, siRNA is incorporated into Argonaute 2 (AGO2) and the RNAi-induced silencing complex (RISC), which checks for complementarity. If the short dsRNA is perfectly matched, AGO2 cleaves the dsRNA into a sense (coding) and an antisense (non-coding) strand. In consequence, RISC, containing the antisense strand, is produced. The RISC and RNA antisense strand are now forming an activated complex that recognizes the target sites and cleaves the target mRNA. A simplistic overview of RNAi mechanism involving siRNA is shown in Figure 1.

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**Figure 1.** A simplistic overview of the RNA interference pathway.

As mentioned before, siRNA can be used as a cure against viruses. There are already some siRNA therapy trials for viral infections. Some of those therapies were tested in vitro and there are still some problems that have to be resolved before starting in vivo experiments. Other therapies have been examined in vivo either on model organisms or infected cell cultures and
gave promising results. Although all of the discussed siRNA aspects are important in making antiviral cures, the efficiency of the cure depends on the virus type and its further properties. Here we discuss some examples of viruses given corresponding siRNA antiviral therapies.

**Cytomegalovirus (CMV) retinitis**

CMV retinitis, also known as cytomegalovirus retinitis, is a DNA virus which causes retinal inflammation that can lead to blindness. The siRNA efficiency in the eye was demonstrated in animal models. The siRNAs can be delivered directly to the target tissue, by means of intravenous injection. The advantages of the local delivery in siRNA therapy is the lower dose required for efficient silencing, because of the reduced exposure to nucleases. Additionally, the risk of siRNA systemic exposure declines by more specific application of a drug. This siRNA therapy has an advantage over the recently given antivirals, because, once siRNA is activated in the cell, it can be active for weeks in non-dividing cells. Thus, the antivirals do not have to be given daily to the patient, but only once in few weeks.

**Avian influenza A (H5N1) virus**

The avian influenza virus H5N1 (AIV), also known as ‘bird flu’, infects the epithelial cells of the upper respiratory tract and lungs. SiRNA which targets the specific conserved regions of the viral genome, such as the PA (polymerase acid), PB1 (polymerase basic 1) and NP (nucleoprotein) gene, could be the solution. Recent tests showed that siRNA could indeed inhibit the viral reproduction. The siRNA that silences the PA and PB1 genes slightly inhibited the viral reproduction, whereas the siRNA targeting NP gene inhibited about 70% of the virus reproduction. This test was performed in cell cultures. To confirm the effectiveness of siRNA, not only in vitro but also in vivo, mice infected with the avian influenza virus were treated with siRNA. Also there was a significant inhibition in viral replication in the lungs of the mice, when they were treated with the siRNA directed to the NP gene. Furthermore, when the mice received treatment with a lethal dose of the H5N1 virus and were treated with the siRNA against the NP gene, 2 of the 8 mice were protected, while in the control group all the mice died.

Based on this study, it can be concluded that siRNA therapy is promising in the treatment of AIV. Targeting conserved viral sequences like the NP gene would reduce the need of inventing new drug almost every year. However, the efficiency of this therapy has to be improved. It can be achieved for example by using combined siRNA targeting both the NP and PA genes.

**Polio virus**

The polio virus is still a severe threat in developing countries, causing poliomyelitis that leads to paralyses and sometimes even death. An experiment performed by Gitlin et al showed almost complete inhibition of the viral replication by siRNA against the polio virus in human cells. In the experiment, two different siRNAs were tested separately. One siRNA silenced the viral protein 3 and the other designed to cleave mRNA coding for the polymerase gene (3D). Tests performed with single siRNA type were less efficient and the protective effect of siRNA on cells infected with the polio virus did not last 24 hours. Twenty four hours after the infections were treated with polio virus and the treatment with the siRNA, 75% of the cells were viable again for the infection. Sequence analysis revealed that silent point mutation had occurred. However, when a mixture of siRNA molecules was used, no escaping viruses were observed.

Another study described combined siRNA cure for persistent poliovirus found total inhibition of viral replication. In this study, performed on human cell culture, combined siRNA was added four times to ensure that every cell infected with the virus would also be transfected with siRNA. After four siRNA treatments, no recovery of virus had been found and thus the cell cultures had been completely cured.

Those experiments showed that the use of combined siRNAs significantly reduced the viral mutation rate. The study also showed that a complete cure of virus with siRNA is

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possible. The main problem is that combined siRNA treatment has to be effectively delivered to the infected cells. Therefore siRNA delivery systems have to be improved.

**Hepatitis C Virus**

The Hepatitis C virus (HCV) causes severe infection in the liver leading to the development of chronic hepatitis, and potentially even cirrhosis and hepatocellular carcinoma. Unfortunately, there is no vaccine available for HCV. Current treatment for HCV, consisting of administering pegylated interferon α and ribavirin, has limited efficiency against certain types of HCV and has many side effects. However, siRNA that specifically targets HCV IRES within the 5' NTR has been tested. An experiment by Chevalier et al. showed that indeed viral replication was inhibited by siRNA. A good candidate for the siRNAs to target against was the HCV IRES region, since this region is essential for the initiation of polyprotein translation and is conserved in many HCV types. The problem with this region was that it harbors strong secondary and tertiary structures, which are difficult for the siRNA to target, though not impossible. Five different siRNAs were proved to inhibit the replication. Even in experiments with replicon-containing cells, the five siRNAs left showed that they strongly inhibited the viral RNA replication and three of these siRNAs even abolished the virus. In contrast, a study done by Yokota et al. showed that some siRNA increased the replication rate instead of decreasing it. This shows that the right target sequence is essential and many siRNAs have to be tested.

Additionally, a perfect base-pairing between the viral RNA and the siRNA is required for the most effective silencing. Only two small mismatches, wobble base-pairs, were tolerated by the most effective siRNAs. Other mismatches were not tolerated; this illustrates the specificity of the siRNA and explains why some HCV infected cell could escape from the siRNA treatment under pressure, like antiviral treatments. This study showed again that a single siRNA treatment is not that effective. To optimize the effectiveness of the siRNA, a multiple siRNA treatment has to be used. The study by Yokota et al. additionally demonstrates that siRNA sequences have to be chosen very precisely to ensure gene silencing.

**Human Immunodeficiency Virus (HIV)**

Belonging to retroviruses, HIV attacks the T-cells of the immune system. HIV infections are currently treated with highly active antiretroviral therapy (HAART). The HAART therapy consists of multiple antiretroviral agents, keeping the amount of HIV low, but it cannot cure the HIV patient nor remove all symptoms. Next to it, antivirals cause various side effects and not all of them can be combined.

The use of siRNAs in HIV therapy brings new perspectives. It could reduce side effects and efficiently silence the HIV. However, HIV is a type of virus where the genome mutates very rapidly and so does the siRNA target site. Son et al. described a treatment of HIV transfected cells which were treated with bi-specific constructs. More than 90% viral inhibition has been observed with two different siRNAs. It suggests that the viral escape can be overcome by using combinatorial siRNAs against conserved sequences especially those that allow wobble-base pairing. This method would be effective and could be used as a complete HIV cure only in the early stage of HIV infection, when the viral genome has not yet integrated into the genome of the host. When the viral genome has integrated into the host genome, the virus cannot be completely cleared. Although it is possible to inhibit viral replication by siRNAs, the viral genome will never be completely removed from the host. Summarizing, when the viral genome has integrated into the host genome, the siRNA therapy would have to be given for the rest of the patient’s life.

A different problem with HIV is that effective siRNA treatment required siRNA delivery to every infected cell in order to effectively kill the virus. If this is not the case, the chance of mutation and escaping is much bigger. In the case of HIV, CCR5, a chemokine receptor, can be a good target for siRNA, because cells with
no CCR5 membrane-proteins are resistant against HIV infection and have no abnormal phenotype. In a test with siRNA against CCR5 and p24, which makes up the viral capsid, the virus production was completely eliminated.\(^\text{15}\) By making cells less susceptible for HIV infection this method could be used in the future as HIV vaccination.

The effect of mutation

The main problem with current therapies against (human life threatening) viruses is the high mutability of the virus. Viral high mutation rates are caused by the leak of DNA polymerases which can find and fix mistakes in the viral genome. The mutations in the viral genome can be fatal for the viral replication, but they also can become a tool in escaping antiviral treatments. Despite high mutation rates in the viral genome, it still seems to be a good target for siRNA therapy. It has been shown by Haasnoot et al.,\(^\text{16}\) that the most effective siRNA will complement with a highly conserved sequence in the viral genome. The chance of a mutation in highly conserved regions is smaller, because it would affect viral fitness. Therefore, targeting conserved regions of the viral genome reduces the possibility of viral escape by mutation. An additional option to reduce viral escape is a combined use of multiple siRNAs, targeting more than one viral genome fragment. There are examples have shown that using two different siRNAs can significantly inhibit or totally block the viral replication.\(^\text{15,17}\) Using combined siRNA, it is harder for the virus to escape silencing, because all sequences targeted by siRNA have to be mutated.\(^\text{16}\) There are, however, more strategies for the virus to escape from the siRNA treatment. First, the virus can replicate in compartments in the cell that are inaccessible to the siRNA, the nucleus for example, and possibly even stay there. Also viral RNA bound to proteins or double-stranded viral RNA are inaccessible for the siRNA-machinery.\(^\text{16}\)

A single point mutation, substitution or deletion seems to be enough for the RNA to not be recognized by siRNA. The deletion can induce an inaccessible RNA structure for the siRNA. When it comes to substitutions, the mismatches can be more or less accepted by AGO2 and the RISC complex if they are at certain positions. Also the mismatches between A:C are less likely to result in loss of silencing than U:G mismatches.\(^\text{17}\) Not all the point mutations are acceptable for the siRNA-machinery. To escape from silencing by siRNA, the point mutations do not have to occur in the specific target sequence. It is possible that mutations outside of targeted region change the secondary structure of the RNA, making it impossible for the siRNA to reach the target sequence of viral RNA.\(^\text{17}\)

Design of siRNA

The design of siRNA has to fulfill some conditions to efficiently inhibit expression of targeted host or virus genes. First of all, previous studies\(^\text{17}\) have established that highly functional siRNA had a G:C content between 32 and 52%. Secondly, low internal stability of siRNA at the 5’ antisense end is also crucial for effective silencing and efficient entry into RISC. To ensure low internal stability, 5’ antisense end has to have a high A:U content. Next to it, the siRNA target sequence cannot contain internal repeats or palindrome sequences. Palindrome sequences form internal fold back structures reducing effective concentration and silencing the potential of siRNA. Therefore, siRNA has to have high internal stability and a good indicator for this is a low melting temperature. Also, the target sequence is important. As already mentioned, the eventual mutations are more accepted at certain positions than others. Therefore an algorithm has to be made in which the crucial positions of the targeted sequences have to be estimated.\(^\text{17}\)

SiRNA stabilization against nucleases

The siRNA therapy faces yet another problem. It is known that siRNA duplexes are highly unstable in serum with a half-life time of minutes.\(^\text{8}\) This rapid siRNA breakdown is caused by the activity of nucleases. To make an efficient siRNA treatment and improve its pharmacokinetic properties, siRNA duplexes have to be resistant against nuclease activity. This can be done by either introducing a phosphorothioate (P=S) backbone linkage at 3’.
ends or by introducing internal 2’ sugar modifications like 2’-O-methyl or 2’-deoxy-2’-fluoro. The structures of modified RNAs are shown in figure 2.

![Figure 2. The modification of sugars in nuclease resistant RNA.](image)

Although the internal sugar modifications are generally well tolerated, the exact modification position within the siRNA duplex seems to be crucial for the preservation of siRNA function. To identify minimal modifications, required for siRNA nuclease resistance, the nuclease degradation fragments of siRNA can be studied. It has been previously established that nuclease resistant siRNAs displayed in vivo improved pharmacokinetics when administered in nuclease rich environments, like blood. However, if siRNAs are delivered to less nuclease rich environments, like lungs, or the exposure to nucleases is minimized by packing siRNA in liposomes, the nuclease stabilization will be reduced to minimum. The nuclease stabilization has also no influence on the stability of siRNA inside the cell. Because the silencing effect caused by siRNA in non-dividing cell can last for weeks, the intracellular siRNA stabilization is not necessary.

**SiRNA delivery to infected cells**

To make siRNA an effective inhibitor of virus replication in in vivo setups, cells already infected with the virus have to be reached. A good delivery system is also required to reduce viral infections. Here we mention several types of delivery systems.

**Local delivery**

Local delivery is the simplest option to deliver naked siRNA. When siRNA is injected or administered near the target tissue, the effective dose of siRNA is lower and in consequence reduces undesirable systemic side effects. However, this method is only efficient in the case of viral infections of easily accessible organs like eye or lungs, but not in the case of liver or kidney viral infections. To target siRNA to other tissues, delivery requires more sophisticated methods.

**Conjugation**

One of the more cell-type specific delivery methods is conjugation. It involves a covalent attachment of siRNA to a targeting molecule. This method is attractive for siRNA delivery, because only one strand of the duplex is active and the targeting molecule can be easily attached to the sense strand without affecting the activity of the antisense strand which is incorporated into the RISC-complex. A well known example in vivo silencing mechanism is the conjugation of siRNA to cholesterol aiming...
to target the liver.\textsuperscript{22, 23} For delivery to specific cell types, siRNA can also be conjugated into RNA aptamers. RNA aptamers are fragments of RNA synthetically designed to recognize cell-type-specific membrane antigens. Aptamers promote specific cellular uptake and cause RNAi mediated target silencing. In theory it is possible to design RNA aptamers for every cell type. Although short aptamers (22-35 bases) do work for local delivery \textit{in vivo}, they will be quickly cleared by the kidney and thus the siRNA half-life would be too short for systemic therapy. This problem could be solved by using longer RNA aptamers (45-55 bases). However, long RNA molecules may be difficult to synthesize on a commercial scale.\textsuperscript{23}

**Liposomes**

Another option to deliver siRNA to cells of concern is by using liposomes, which consist of a phospholipid bilayer enclosing the aqueous compartment with siRNA. Liposomes can fuse with a specific cell membrane, which is determined by the composition of the phospholipid bilayer of the vesicle. The most successful example of delivery siRNA using liposomes is targeting the liver tissue. Although a lipid based delivery system of siRNA represents a promising perspective, there are primary cell types, like lymphocytes, which are resistant to lipid-mediated transfection.\textsuperscript{8}

**Polyethylenimine I (PEI)**

It is also possible to transfer siRNA using cationic peptides and polymers. siRNA interacts with these positively charged peptides by ionic interactions between negatively charged phosphate backbones to form stable nanoparticles. The most impressive example of this type of siRNA delivery is PEI. PEI has a synthetic, linear or branched structure with high cationic charge densities. The polyplexes formed by PEI and siRNA are thought to interact with the cell surface electrostatically and are transported into the cells by endocytosis. Subsequently, in the endosome, PEI acts like a proton sponge, disrupting endosomal pH, causing an increased influx of protons and water into the endosome. In consequence, the endosome swells up and finally the polyplexes of siRNA and PEI are released into the cytoplasm.\textsuperscript{24} Although this delivery method turned out to be successful in against influenza infection in mice,\textsuperscript{23} it turned out to be extremely toxic in higher doses. Currently, optimizing PEI physical structure to improve \textit{in vivo} siRNA delivery is in progress.

In conclusion, that siRNA therapy has serious advantage over other antiviral therapies. However, the siRNA target sequence has to be homologous enough to inhibit viral replication. It cannot overlap with mRNAs coding for host cell essential structures and is protected from digestion by nucleases. In addition, it is obvious that several aspects of siRNA therapy have to be improved, such as a delivery system or virus target sequences. Nevertheless, the arising possibilities of siRNA therapy as antiviral agents or vaccination are astonishing.

**REFERENCES**